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TDP43 proteinopathy is associated with aberrant DNA methylation in human amyotrophic lateral sclerosis

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Background: Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by motor neurone (MN) degeneration and death. ALS can be sporadic (sALS) or familial, with a number of associated gene mutations, including *C9orf72* (C9ALS). DNA methylation is an epigenetic mechanism whereby a methyl group is attached to a cytosine (5mC), resulting in gene expression repression. 5mC can be further oxidized to 5-hydroxymethylcytosine (5hmC). DNA methylation has been studied in other neurodegenerative diseases, but little work has been conducted in ALS. **Aims**: To assess differences in DNA methylation in individuals with ALS and the relationship between DNA methylation and TDP43 pathology. **Methods**: *Post mortem* tissue from controls, sALS cases and C9ALS cases were assessed by immunohistochemistry for 5mC and 5hmC in spinal cord, motor cortex and prefrontal cortex. LMNs were extracted from a subset of cases using laser capture microdissection. DNA from these underwent analysis using the MethylationEPIC array to determine which molecular processes were most affected. **Results**: There were higher levels of 5mC and 5hmC in sALS and C9ALS in the residual lower motor neurones (LMNs) of the spinal cord. Importantly, in LMNs with TDP43 pathology there was less nuclear 5mC and 5hmC compared to the majority of residual LMNs that lacked TDP43 pathology. Enrichment analysis of the array data suggested RNA metabolism was particularly affected. **Conclusions**: DNA methylation is a contributory factor in ALS LMN pathology. This is not so for glia or neocortical neurones.

Keywords: amyotrophic lateral sclerosis, DNA hydroxymethylation, DNA methylation, epigenetics, motor neurone disease

Introduction

Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is the most common motor neurone disease. It is characterized by progressive loss of motor function brought on by degeneration and death of motor neurones (MNs) and was first described in 1869 [1]. Degeneration is seen in both upper motor neurones (UMN) and lower motor neurones (LMN), leading to muscle weakness, spasticity and atrophy, with death typically occurring

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within 3 years after disease onset. The incidence of ALS is 1.5–2 per 100 000 people, per year, with a prevalence of 5–7 per 100 000 people worldwide [2]. The causes of ALS are not completely elucidated. Risk factors include having an immediate family member diagnosed with the disease, a lifestyle with vigorous prolonged exercise [3] and advanced age [4]. Pathologically, ALS is characterized by lower motor neurone loss, with a subset of residual motor neurones showing so-called TDP43 proteinopathy: There is a loss of TAR DNA-binding protein 43 kDa (TDP43) from its normal location in the nucleus, with the concomitant formation of cytoplasmic aggregates of ubiquitylated, phosphorylated, cleaved TDP43 in the cytoplasm [5,6].

ALS is classified as either familial ALS (fALS) or sporadic ALS (sALS): fALS is defined as having a family history of ALS, potentially with a known associated gene mutation. sALS refers to cases with no known family history of the disease. Sporadic cases account for the majority of ALS, (90–95%). fALS makes up 5–10% of diagnoses, defined as specific associated genetic dominant inheritance of a mutated gene or having first- or second-degree family member with the disease, although some nonautosomal dominant cases have been reported [7].

Mutations in the chromosome 9 open-reading frame (*C9orf72*) gene are found in 37% of fALS cases and 7% of sALS cases, making it the most commonly mutated gene in ALS [8]. TDP43 is coded for by the *TARDBP* gene [9]. Mutations in the *TARDBP* gene account for approximately 1% of sALS cases and 4% of fALS cases [10]. TDP43 proteinopathy as described earlier is seen in almost all cases of sALS as well as most genetic forms (including C9ALS, and ALS due to *TARDBP* mutations). TDP43 has a number of roles in normal cellular function (see below) and has been posited to have many pathological roles in ALS, including changes in protein stability and degradation, impaired cytoskeletal function and altered homeostasis of DNA- and RNA-binding proteins [11].

TDP43 is mainly localized to the nucleus, but also shuttles to and from the cytoplasm. Within the nucleus, TDP43 regulates splicing of both noncoding and protein-coding genes involved in neuronal survival [12] and mitochondrial homeostasis maintenance by regulating mitochondrial transcript processing [13]. In the cytoplasm, TDP43 is also involved in transcription regulation, RNA transport and stress granule formation in response to cellular stress [14].

TDP43 is can also bind DNA and was first identified as a co-factor that binds to a regulatory element in the long terminal repeat of the human immunodeficiency virus type 1 (HIV-1), resulting in in vitro transcription being repressed from this element [9].

Aside from the possible role of TDP43 in DNA processing as described above, there are other reasons why study of DNA pathology in ALS may be of interest. Here, we focus on DNA methylation. DNA damage in this series of ALS brains has been reported elsewhere [15].

Epigenetics: DNA methylation

Epigenetic changes are defined as stable alterations that are made to either DNA or histone proteins that alter gene expression, but do not affect the base sequence. Epigenetic mechanisms are involved in gene expression regulation and are required for specific cell-type differentiation [16]. DNA methylation is the process of attaching a methyl group to a DNA base – usually a cytosine that is followed by a guanine, so-called CpG sites. This can result in gene silencing if the methylation event prevents the binding of transcription factors to a gene promoter by changing the chromatin structure. DNA methylation has also been shown to be involved in promoting genomic stability [17].

DNA methyltransferases (DNMTs) are responsible for the attachment of methyl groups to cytosine nucleotides in DNA and have two classes: *De novo* DNMTs are able to newly methylate cytosines, setting up DNA methylation patterns (DNMT3a and DNMT3b), whereas maintenance DNMTs maintain these DNA methylation patterns (DNMT1).

Cytosine methylation results in the formation of 5-methylcytosine (5mC). This can be oxidized by ten-eleven translocation (TET) enzymes to 5-hydroxymethylcytosine (5hmC). Further oxidation results in further demethylation into 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) [18]. Terminal deoxynucleotidyl transferase (TdT) or thymine DNA glycosylase (TDG) convert 5fC and 5caC back to unmethylated cytosine by base excision repair [19].

The presence of 5mC is implicated in gene expression repression, synaptic plasticity, gene imprinting, chromatin structure maintenance and X-chromosome inactivation. It is enriched in genes encoding proteins with

function in neurones and other nervous system cells [20]. 5hmC levels have been shown to be generally present at 10% of the levels of 5mC in the genome [21], but with higher levels present in the CNS [22]. 5fC and 5caC levels are even less abundant in the genome than both 5mC and 5hmC [18].

DNA methylation in ALS

There are good reasons to suppose that DNA methylation levels are altered in ALS. First, age is one of the most important risk factors for ALS [4]. This raises the question of which age-related changes cause susceptibility for ALS. It is well established that DNA methylation alters with age. First, the so-called 'epigenetic drift' describes the tendency towards less consistent methylation with age, with an overall net reduction in methylation across the genome [23]. Second, epigenetic age estimators, referred to as 'epigenetic clocks', indicate how biologically aged a specific tissue or blood is in comparison to the chronological age of an individual based on the pattern of gene methylation seen. Horvath's clock is the most well-known of the epigenetic clocks [24]. Such epigenetic age estimators use sets of CpGs to estimate the age of a tissue. Epigenetic clock algorithms have been used to study age-related neurodegenerative conditions, including Alzheimer's disease (AD), dementia, Huntington's disease (HD) and Parkinson's disease [25], with findings suggesting an accelerated epigenetic age in those with neurodegenerative conditions compared to those who were neurologically healthy.

DNA methylation changes have been observed for neurodegenerative diseases using other technologies as well. Thus, for example altered methylation status has been demonstrated variously by immunohistochemistry for 5mC and 5hmC, gas chromatography/mass spectrometry, and the Illumina 450k array platform in Alzheimer's disease, dementia with Lewy bodies, Huntington disease and frontotemporal lobar degeneration [26-30].

The first study to investigate the role DNA methylation played in ALS was an epigenome wide association study (EWAS) [31]. Frontal cortex from ten sALS patients and ten controls underwent DNA methylation analysis using Affymetrix GeneChip Human Tiling 2.0R arrays. A number of genes were found to be differentially methylated, with pathway analysis showing altered DNA methylation in genes involved in calcium homeostasis, oxidative stress and neurotransmission.

A second study to use EWAS in ALS, has shown global increases in 5mC and 5hmC levels in *post mortem* sALS spinal cord, but not in blood [32]. This suggests that the DNA methylation profiles in the CNS differ from those of blood. This highlights that blood biomarkers may not be a good indicator of crucial DNA methylation changes in ALS and illustrates cell- and tissue-specificity of DNA methylation patterns.

A key issue is the ability to assess individual cell types: Methylation is a mechanism that is key to cellular differentiation, and simultaneous analyses of different tissue types can find different methylation patterns in any given disease. Thus, each cell type within the CNS would be expected to have different DNA methylation patterns [16]. Furthermore, ALS is characterized by a loss of neurones and myelin with a concomitant astrogliosis and microgliosis [33]. Therefore, differing methylation profiles will be seen between control and disease tissue purely because of the shift in cellular composition. It is therefore necessary to determine each cell type's specific methylation pattern in relation to disease.

In summary, the literature to date reveals: First, age is a key risk factor for ALS, raising the question as to what age-related changes are relevant to the development of disease. Second, DNA methylation profiles alter with age and a number of other neurodegenerative diseases. Third, the DNA methylation pattern seen can be both cell- and tissue-specific. Finally, while RNA-based pathology has been intensively researched with respect to ALS, DNA-related pathology has been relatively neglected. We have therefore addressed this in *post mortem* CNS samples from individuals with sporadic ALS and ALS due to *C9orf72* mutations. Using immunohistochemistry on *post mortem* CNS samples, we have assessed levels of methylation and hydroxymethylation in glia, lower motor neurones, upper motor neurones and pyramidal cells of the prefrontal cortex. Using Illumina EPIC gene chips, we have assessed which biological processes are predominantly affected in lower motor neurones.

Materials and Methods

Formalin-fixed, paraffin-embedded (FFPE) human *post mortem* spinal cord, motor cortex and anterior frontal

cortex taken from the middle frontal gyrus was obtained from the Sheffield Brain Tissue Bank (SBTB). For this study, sections from were used from three groups: controls, sporadic ALS (sALS) and ALS caused by *C9orf72* mutations (C9ALS). Groups were age- and sex-matched, as far as was possible. Ethical approval for this study has been granted by the Management Committee of SBTB, which in turn has ethical approval to provide tissue for research under the provision to act as a Research Tissue Bank as approved by the Scotland A Research Ethics Committee (ref. 08/MRE00/103).

Immunohistochemistry

IHC was carried out for TDP43, 5mC and 5hmC. Sections were subjected to IHC using the standard ABC technique (Leica, UK). Positive controls were used to assess the efficiency of staining. Either negative controls or IgG isotype controls were conducted, with the primary antibody step omitted from one section in each IHC run. Antibody specificity checks were carried out for both 5mC and 5hmC to ensure that any immunopositive staining observed was specific: Preabsorption of the antibodies was carried out, as well as DNase treatments.

Sections were first blocked for 30 min at room temperature (RT), followed by primary antibody incubation (see Table S1 for providers and conditions). The secondary antibody was applied for 30 min at RT, followed by ABC incubation for 30 min at RT. Immunopositive staining was visualized using DAB.

Cell counting

The immune-stained slides were digitized using the Hamamatsu NanoZoomer XP, visualized by NDP.view2 digital pathology software (Hamamatsu Photonics, Japan) and counted by the first author (CAM, Figure 1). Prior to this, Cohen's kappa coefficient [34] was used to assess the reliability of this method of assessing nuclear immune-positivity for neurons and glia: A series of 140 cells per assessment were classified by CAM and a qualified diagnostic neuropathologist (JRH) revealing all $\kappa \geq 0.81$ indicating a high level of reproducibility. Two rounds of cell counting and assessment were performed.

First, in order to assess differences in methylation between ALS and control cases, counts were made of neuronal and glial nuclei that were positive and negative for 5hmC and 5hmC irrespective of TDP43 status. A larger number of cells were counted for this analysis as there was no requirement for visualization in adjacent slides. This was performed in the following structures (See Tables S2–S4 for demographics):

(1). Anterior horn, lateral corticospinal tract and dorsal column in the spinal cord (n = 10 controls, 10 sALS cases and 10 C9ALS);
(2). Neocortex and underlying white matter of the motor cortex (n = 8 controls, 21 sALS cases and 12 C9ALS cases);
(3). Neocortex and underlying white matter of the middle frontal gyrus/anterior frontal cortex (n = 11 controls, 30 sALS cases and 12 C9ALS cases).

Second, in order to assess the effect of TDP43 proteinopathy (nuclear loss of TDP43) on lower motor neurones in ALS cases, sequential, adjacent sections of spinal cord were immunostained for TDP43, 5mC and 5hmC. The digitized whole-slide images were aligned such that the same neurone nucleus could be simultaneously assessed for either both TDP43 and 5mC or TDP43 and 5hmC (Figure S1). MN nuclei were graded to assess nuclear immunopositive and immunonegative staining. A total of 200 cells were assessed for either 5mC and TDP43 or 5hmC and TDP43 in 20 ALS cases (10 sALS and 10 C9ALS cases, demographics as per Table S2) were assessed.

Statistical analysis was carried out using either SPSS version 24 or 25. An inter-rater reliability test (Cohen's Kappa) was conducted to assess the reliability, validity and reproducibility of cell counting. This calculation is a chance-corrected version of the observed agreement. High agreement between observers indicates diagnosis consensus as well as consensus on the interchangeability and reliability of the ratings. One-way ANOVA was also used to assess any potential relationships between 5mC/5hmC status in the MNs and TDP43 pathology. One-way ANOVA was also used to test differences in the percentage of cells that were immunopositive for 5mC and 5hmC between the three groups (controls, sALS and C9ALS). Intergroup comparisons are also presented as barcharts with error bars indicating one standard deviation.

MethylationEPIC array

Formalin-fixed, paraffin-embedded human *post mortem* spinal cord was obtained from the SBTB. For this study, sections were from three groups: controls, sALS and C9ALS, with n = 6 for each cohort (total n = 18,

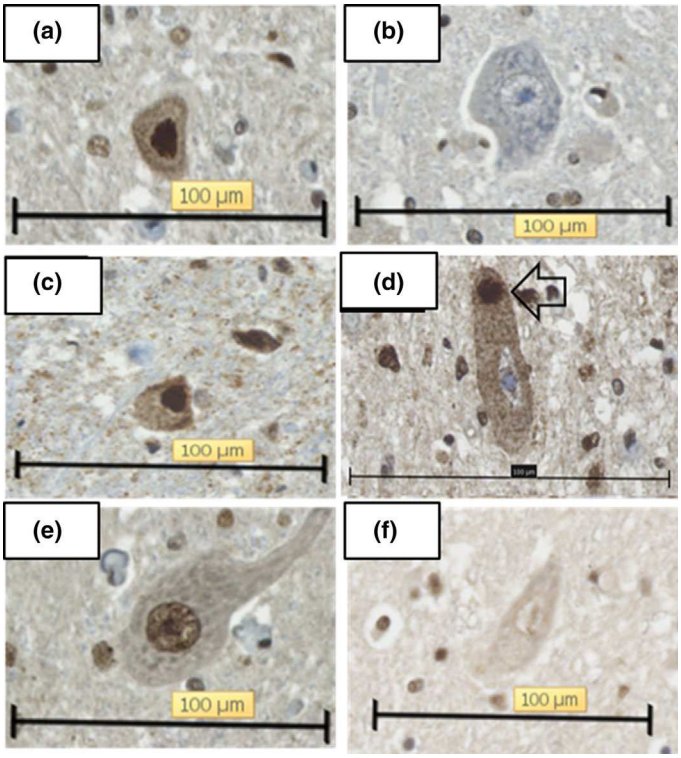

Figure 1. Representation of grading system used for slide analysis in TDP43 and 5mC/5hmC sequential staining. Images taken at ×80 magnification, scale bar represents 100 µm. (**a**) MN nucleus displaying 5hmC immunopositive staining, (**b**) MN nucleus displaying 5hmC immunonegative staining, (**c**) MN nucleus displaying TDP43 immunopositive staining, (**d**) MN nucleus displaying TDP43 immunonegative staining, with TDP43-positive inclusion in cytoplasm (arrow), (**e**) MN nucleus displaying 5mC immunopositive staining, (**f**) MN nucleus displaying 5mC immunonegative staining.

Table S5). 10 µm sections of tissue were mounted onto uncharged slides, followed by dewaxing and rehydration. Lower motor neurones (LMN) were visualized using toluidine blue (0.01%), before being dehydrated, cleared in xylene and air dried for 1hr. LMNs were extracted from tissue using laser capture microdissection (LCM). The Arcturus VERITAS™ LCM system (Applied Biosystems), in conjunction with Arcturus Capsure Macro LCM Caps (Life Technologies), was used with the following settings: 30 µm spot size, 70 mW laser power, 2500 µsec pulse, one hit, 200 mV laser intensity and 0 msec delay. Approximately 10 000 cells were collected per case on six caps. DNA was extracted using the Zymo quick-DNA™ FFPE kit (Zymo Research), as per manufacturer's protocol. Bisulphite conversion was then performed using the Zymo EZ DNA Methylation-Direct Kit (Zymo Research). A NanoDrop 1000 spectrophotometer (Thermoscientific) and Agilent HS DNA chip were used to assess DNA quantity and quality. DNA methylation was assessed using the Illumina® Infinium® MethylationEPIC BeadChip and imaged on an Illumina® HiSeq® 2500 scanner.

Bioconductor and the programming language R (version 3.5.0), using the RnBeads package in conjunction with a GUI vignette, known as the RnBeads Data Juggler, were used to analyse the BeadChip data [35,36]: IDAT files containing the data collected from the scanned BeadChips along with a.csv file containing case details were loaded into Data Juggler. All analysis was sorted based on differentially methylated promoters, with $\alpha = 0.05$. This was then further sorted by identifying which promoters were classed as either hypomethylated or hypermethylated in ALS *vs.* control. Gene ontology analysis was carried out as part of the RnBeads analysis. GO terms were considered significant at $P < 0.01$ following Benjamini–Hochberg correction.

Results

DNA methylation and hydroxymethylation is higher in lower motor neurones (LMNs) affected by ALS

The relationship between ALS pathology and methylation was explored by counting the overall percentage of LMNs in

the anterior horn of spinal cord that were positive for 5mC and 5hmC in controls and two ALS groups (sALS and C9ALS). The percentage of LMN that were positive for 5mC and 5hmC was significantly lower in controls (76.3 ± 7.1% for 5mC; 81.6 ± 5.7% for 5hmC) than both sALS (89.5 ± 3.3%, $P = 0.01$ for 5mC; 90.4 ± 3.8%, $P = 0.001$ for 5hmC) and C9ALS (90.9 ± 3.1%, $P = 0.003$ for 5mC; 90.7 ± 4.7%, $P = 0.0007$ for 5hmC, Figure 2). No difference was observed between the two ALS groups (sALS and C9ALS; $P \geq 0.53$).

5mC and 5hmC levels in spinal cord glia do not differ between controls and ALS

Counts of 5mC and 5hmC positive and negative glia were similarly carried out in the anterior horn, lateral corticospinal tract and dorsal column of the spinal cord. In contrast to lower motor neurones, there were no significant intergroup differences in the levels of glial methylation and hydroxymethylation in any of the regions investigated ($P \geq 0.05$).

There is minimal difference in neuronal or glial 5mC or 5hmC in motor cortex and anterior frontal cortex in ALS.

Having demonstrated altered methylation and hydroxymethylation in the lower motor neurones (but not glia) of the spinal cord, we next investigated whether this would be observed in the forebrain. For the motor cortex, there were no intergroup differences in levels of methylation or hydroxymethylation for neurones ($P \geq 0.22$) or glia ($P \geq 0.17$).

For the anterior frontal cortex (middle frontal gyrus), only very minor changes were observed in neurones: There was slightly greater 5mC labelling in sALS (mean 75.8%, SD 6.0%) than controls (mean 71.7%, SD 4.3%; $P = 0.04$). There was slightly greater 5hmC labelling in sALS (mean 76.3%, SD 5.2%) than C9ALS (mean 72.3%, SD 3.4%; $P = 0.02$). Other intergroup comparisons for 5mC and 5hmC were nonsignificant ($P \geq 0.20$). For the anterior frontal cortex glia, there were no significant intergroup differences for 5mC or 5hmC, (all $P \geq 0.24$).

There is greater DNA methylation in lower motor neurones than upper motor neurones.

Having demonstrated that residual spinal cord lower motor neurones in ALS had greater levels of methylation and hydroxymethylation than controls, but that there were no such intergroup differences seen in the upper motor neurones of the precentral gyrus or the neurones of the anterior frontal cortex. We were thus interested to know if forebrain and spinal cord neurones differed in their levels of DNA methylation. There were higher levels of DNA methylation and hydroxymethylation in the spinal cord than in motor and anterior frontal cortices (all $P \leq 2 \times 10^{-10}$) across the entire cohort (control, sALS and C9ALS cases combined). This was also seen when the analysis was restricted to cases from the control cohort alone ($P \leq 0.0003$).

TDP43 pathology is associated with lower DNA methylation and hydroxymethylation

Having demonstrated greater levels of methylation and hydroxymethylation in the lower motor neurones in sALS and C9ALS, we proceeded to study the effect of TDP43 pathology on these cells in ALS: Individual

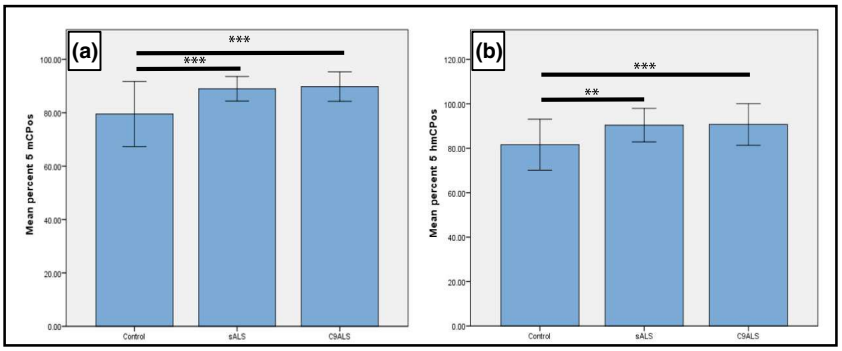

Figure 2. Differences in global levels of 5mC and 5hmC in motor neurones of the anterior horn are present between control and disease (mean, with error bars corresponding to one standard deviation). $n = 10$ for each group, total $n = 30$. (**a**) Significantly higher 5mC is observed in sALS and C9ALS cases than controls ($P = 0.0001$ and 0.0002 respectively). No difference was observed between the two diseased groups ($P = 0.48$). (**b**) Significantly higher 5mC is observed in sALS and C9ALS cases than controls ($P = 0.0011$ and 0.00072 respectively). No difference was observed between the two diseased groups ($P = 0.88$).

lower motor neurone nuclei were identified in adjacent sections and assessed for TDP43 (loss of labelling corresponding to pathology). Each neurone was then found on the adjacent section and assessed for either 5mC or 5hmC. In neurones with normal, TDP43-positive nuclei, there were high levels (mean ± SD) of 5mC and 5hmC (72.7% ± 15.5 of nuclei were positive for 5mC; 86.9% ± 6.0 positive for 5hmC). However, a significantly lower percentage of MN nuclei displayed immunopositivity for 5mC and 5hmC in pathological neurones where nuclear TDP43 was absent (27.7%±37.9, $P = 0.00002$ for 5mC, and 50.7% ± 16.6, $P < 0.00001$ for 5hmC, Figure 3).

ALS has significantly differentially methylated promoter regions

Having found differential methylation in lower motor neurones in ALS, we next wished to gain some indication of which processes were most affected by this. MNs were extracted from the anterior horn of human *post mortem* cervical spinal cord by laser capture microdissection and analysed by Illumina® Infinium® MethylationEPIC BeadChip ($n = 6$ in each group, total $n = 18$; data available at the University of Sheffield online research data hub, https://orda.shef.ac.uk/, with the access code 10.15131/shef.data.12058110).

First, this revealed that there was a greater number of methylated sites in C9ALS cases (mean of 419 966 sites methylated, SD = 98 783) than controls (mean of 263 207 methylated sites, SD = 38 856; $P = 0.011$). There was an intermediate number of methylated sites in sALS cases (mean of 309 017 sites methylated, SD = 57 157), that was not significantly different from either the control or C9ALS groups.

Next, the analysis focussed more specifically on methylation of known gene promoters – a subset of the number of methylation sites assessed by the array. A total of 732 gene promoters were identified as being significantly differentially methylated in ALS compared to controls at $P \leq 0.05$): 402 promoters were hypermethylated in ALS, and 330 were hypomethylated in ALS. The degree of dysmethylation was more profound in the hypermethylated promoters, with a mean increase in methylation of 21.8% in ALS compared to controls, with hypomethylated promoters showing a mean decrease in methylation of 9.4% in ALS *vs.* controls. Of the 732 promoters, 378 were identified as protein-coding according to the GeneCards database [37].

RNA metabolism dysregulation in ALS

Gene ontology (GO) analysis was conducted as part of the RnBeads analysis in order to identify which biological processes were most affected by dysmethylation of protein coding gene promoters in ALS. This was performed separately for hypermethylated and hypomethylated protein-coding gene promoters with processes considered significant at $P < 0.01$ following Benjamini–Hochberg correction. A number of pathways involved with neurodegeneration were found. For hypermethylation, 30 GO terms were enriched, many of which implicate RNA processing, nucleic acid processing and RNA splicing. For hypomethylated genes, only eight GO terms were enriched, the most significant of which related to urea homeostasis (Table 1).

Discussion

We have performed an investigation into DNA methylation in *post mortem* CNS tissues in amyotrophic

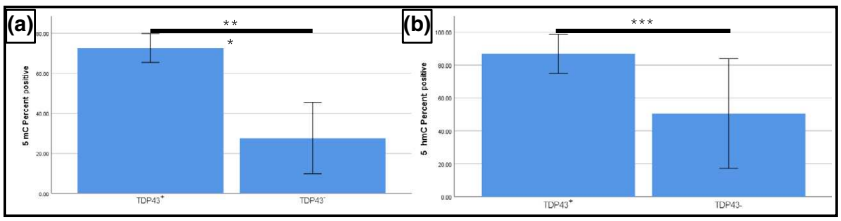

Figure 3. 5mC and 5hmC loss from the nucleus of motor neurones is related to TDP43 motor neurone nuclear loss (mean, with error bars corresponding to one standard deviation). (**a**) 5mC. High levels of methylation were present in motor neurone nuclei positive for TDP43. However, a significantly lower percentage of motor neurone nuclei displayed immunopositivity for 5mC when nuclear TDP43 was lost, $P = 0.00002$. (**b**) 5hmC. High levels of methylation were present in motor neurone nuclei expressing TDP43. However, a significantly lower percentage of motor neurone nuclei displayed immunopositivity for 5hmC when nuclear TDP43 was lost, $P < 0.00001$.

Table 1. Gene ontology enrichment analysis for significantly differentially hypermethylated and hypomethylated promoters identified in MethylationEPIC analysis

GOMFID	*P*-value	*Term*
Hypermethylated		
GO:0000395	0	mRNA 5'-splice site recognition
GO:0000245	0	spliceosomal complex assembly
GO:0071826	1.00E-04	ribonucleoprotein complex subunit organization
GO:0022613	1.00E-04	ribonucleoprotein complex biogenesis
GO:0000377	2.00E-04	RNA splicing, via transesterification reactions with bulged adenosine as nucleophile
GO:0098884	4.00E-04	postsynaptic neurotransmitter receptor internalization
GO:0008380	5.00E-04	RNA splicing
GO:0006397	9.00E-04	mRNA processing
GO:0110077	0.0016	vesicle-mediated intercellular transport
GO:1904906	0.0016	positive regulation of endothelial cell-matrix adhesion via fibronectin
GO:0070194	0.0032	synaptonemal complex disassembly
GO:0071840	0.0041	cellular component organization or biogenesis
GO:1905180	0.0048	positive regulation of cardiac muscle tissue regeneration
GO:0043312	0.0061	neutrophil degranulation
GO:0106027	0.0063	neuron projection organization
GO:0034622	0.0063	cellular protein-containing complex assembly
GO:0090673	0.0064	endothelial cell-matrix adhesion
GO:1903416	0.0064	response to glycoside
GO:0098657	0.0067	import into cell
GO:0042119	0.0067	neutrophil activation
GO:0030100	0.0074	regulation of endocytosis
GO:0000320	0.008	re-entry into mitotic cell cycle
GO:0016321	0.008	female meiosis chromosome segregation
GO:0045321	0.0081	leucocyte activation
GO:0031503	0.0086	protein-containing complex localization
GO:0002275	0.0089	myeloid cell activation involved in immune response
GO:0002444	0.0094	myeloid leucocyte-mediated immunity
GO:0097500	0.0096	receptor localization to nonmotile cilium
GO:0099505	0.0096	regulation of presynaptic membrane potential
GO:0022607	0.01	cellular component assembly
Hypomethylated		
GO:0097274	0.0011	Urea homeostasis
GO:0071947	0.0027	Protein deubiquitination involved in ubiquitin-dependent protein catabolic process
GO:0023041	0.0037	Neuronal signal transduction
GO:0015867	0.0048	ATP transport
GO:0036444	0.0053	Calcium import into the mitochondrion
GO:0048681	0.0064	Negative regulation of axon regeneration
GO:0015865	0.0075	Purine nucleotide transport
GO:0022038	0.0085	Corpus callosum development

lateral sclerosis. Using immunohistochemistry, we found that there were greater levels of methylation and hydroxymethylation in the residual lower motor neurones of both sporadic ALS and C9ALS compared to the lower motor neurones of controls.

Next, in lower motor neurones from ALS cases, we found that neurones with pathological loss of TDP43 from the nucleus, had lower levels of both 5mC and 5hmC, than in neurones with normal nuclear TDP43, suggesting a relationship between TDP43 proteinopathy and DNA methylation. It is worth noting that approximately 90% of residual neurones retain nuclear TDP43 in our cohort.

This pattern could imply either: (i) methylation is initially elevated in neurones in ALS but drops away when TDP pathology develops or (ii) TDP43 pathology favours neurones with lower methylation and leads to death of neurones with lower levels of pathology, leaving behind neurones with greater levels of methylation. The direction of causation remains unknown. It is possible that: (i) ALS and TDP43 proteinopathy cause dysmethylation; (ii) dysmethylation precipitates or

facilitates ALS and TDP43 proteinopathy or (iii) a third factor precipitates both dysmethylation and ALS/TDP43 proteinopathy.

In contrast to the spinal cord, only minor intergroup differences in neuronal methylation or hydroxymethylation levels were found in motor or anterior frontal cortex. One possible reason for this is that TDP43 pathology affects a lesser percentage of neurones in the forebrain than in LMNs; another is the potential role of selective vulnerability. In this context, it is interesting that levels of neuronal DNA methylation and hydroxymethylation were greater in the spinal cord than the forebrain (motor and anterior frontal cortices): It is possible that lower motor neurones require higher methylation levels for ongoing metabolism such that lower levels increase the risk of degeneration, in the form of TDP43 proteinopathy. Resolving the patterns of causation will require functional studies.

Previous studies have suggested that there may be DNA methylation changes in ALS, but all have tended to focus on tissue homogenates. Thus, Figueroa-Romero *et al.* [32] conducted a study of DNA methylation on sporadic ALS spinal cord homogenates and found an increase in DNA methylation using ELISA for 5mC and 5hmC. However, we (above) and others (see introduction) have demonstrated cell-specific DNA methylation changes. As ALS is characterized by neuronal loss, astrogliosis and microgliosis [33], the relative proportion of different cells in ALS and control tissue differ. Thus, any differences in overall methylation in homogenized tissue may reflect different cellular populations more than different levels of methylation within those cell types. This raises the possibility that hypermethylation may be neuroprotective. This is an enticing possibility as there are already pharmacological agents that can reduce DNA methylation as well as ongoing interest in developing agents to boost DNA methylation [38]. Furthermore, DNA methylation is heavily dependent on the so-called 'one-carbon' amino acid metabolic pathway: This pathway is reliant on B vitamins, obtained from dietary sources, and provides a mechanism for the transfer of one-carbon groups (which would be too volatile if not bound other co-factors) between molecules [39]. It is therefore relevant that ALS is associated with elevated levels of homocysteine (a biomarker of B vitamin deficiency) and that B vitamins can rescue a motor neurone cell model from homocysteine-related toxicity [40].

A limitation of this study is that different glial cell populations were pooled as opposed to not separated into astrocytes, microglia and oligodendrocytes. It is probable that there are differences in methylation in these cell populations. It is also possible that these glial types will have different methylation responses to ALS, such that pooling the population will mask any pathological changes that may exist.

Analyses using the MethylationEPIC platform were performed in a subset of cases in an effort to elucidate which biological processes may be most affected by DNA methylation changes in ALS. Greater numbers of methylated sites were observed in the residual lower motor neurones of the C9ALS cohort than the control cohort, reflecting finding of greater percentages of 5mC- and 5hmC- positive nuclei described above. The sALS group had intermediate numbers of methylated sites. With respect to individual genes, 402 gene promoters were hypermethylated in ALS, and 330 were hypomethylated in ALS. Gene ontology analysis of the hypermethylated promoters in combined ALS heavily implicated RNA metabolism with a particular emphasis on RNA splicing. This correlates well with our previous work using exon arrays on lower motor neurones obtained by laser capture microdissection tissue from *post mortem* material from the same brain bank [41]: In this study, there was considerable evidence for aberrant mRNA splicing, as well as significant enrichment of gene ontology terms among genes that were differentially expressed. This study of mRNA expression also provides some validation for the findings from DNA methylation.

We have achieved a level of validation of the MethylationEPIC platform results by demonstrating that the genes that are differentially methylated in ALS are involved in RNA processing (including splicing). However, one limitation of our study is that we have not managed to establish a protocol for demonstrating cell- and gene-specific differential methylation by means other than the array analysis of bisulphite-converted DNA.

The finding of aberrant methylation in spinal cord lower motor neurones but not glia or forebrain neurones contrasts with work on DNA damage and senescence markers: There does not appear to be evidence of DNA damage in lower motor neurones or forebrain neurones although an excess of senescence markers is seen in forebrain glia [15]. This raises the possibility

that different regions and cell types vary in which form of DNA pathology develops in ALS.

Conclusion

We have studied methylation and hydroxymethylation in brain and spinal cord using immunohistochemistry for 5mC and 5hmC in sporadic and *C9orf72*-related amyotrophic lateral sclerosis and controls (sALS and C9ALS respectively). The residual lower motor neurones of the spinal cord had higher levels of methylation and hydroxymethylation than the lower motor neurones of controls. There was no difference between sALS and C9ALS and no intergroup differences in glial cell methylation. Furthermore, only small intergroup differences (if any) were seen in the neurones of the motor cortex and middle frontal gyrus. This suggests that the most significant changes in DNA methylation specifically affect lower motor neurones and not other cell types or neurone types.

Within the two ALS groups, when lower motor neurones with and without TDP pathology (loss of nuclear TDP43) were compared, residual lower motor neurones with TDP43 proteinopathy had lower levels of 5mC and 5hmC than residual neurones with normal nuclei that were positive for TDP43.

Thus, in the lower motor neurones, there are two contrasting effects: ALS cases have higher levels of methylation, whereas within ALS cases, the minority of cells with TDP43 proteinopathy (nuclear loss) have lower levels of methylation. This may suggest that initially, DNA methylation is elevated in ALS, but drops away markedly when TDP43 pathology develops in a cell. Alternatively, TDP43 pathology followed by cell death affects cells with lower levels of DNA methylation, such that more hypermethylated cells are left behind.

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Authors' contributions

CAM, JCK, PRH and JRH involved in study design. CAM, ES and JRH performed immunohistochemistry and data gathering. CAM, PRH and JRH carried out data analysis. PJS, JCK and JRH involved in subject recruitment, clinical and neuropathological diagnosis and characterization. PJS, JK and JCK involved in genetic characterization. CAM provided the original draft of the manuscript. All other authors contributed to further revisions, suggestions and drafts. All authors read and approved the final manuscript.

Disclosure

The authors declare no conflicts.

Data availability statement

The data that support the findings of this study are available in the University of Sheffield online research data hub at https://orda.shef.ac.uk/, reference number 10.15131/shef.data.12058110).

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Motor neurone nuclei of the anterior horn in cervical spinal cord can be visualized and graded for 5mC and TDP43 immunopositivity in sequential sections.

Table S1. Primary antibody details.

Table S2. Demographic details for samples used in spinal cord DNA methylation and hydroxymethylation studies.

Table S3. Case details for samples used in motor cortex DNA methylation and hydroxymethylation studies.

Table S4. Case details for samples used in anterior frontal cortex DNA methylation and hydroxymethylation studies.

Table S5. Promoters identified as differentially methylated by Illumina® Infinium® MethylationEPIC BeadChip.

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